Identification of Hub Genes related to the Progression of Bladder Cancer by an Integrated Bioinformatics Analysis

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Research Article

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Abstract

BACKGROUND and OBJECTIVE: A better understanding of the molecular mechanisms underlying bladder cancer is necessary to identify candidate therapeutic targets.

METHODS: We screened for genes associated with bladder cancer progression and prognosis. Publicly available expression data were obtained from TCGA and GEO to identify differentially expressed genes (DEGs) between bladder cancer and normal bladder tissues. Weighted co-expression networks were constructed, and Gene Ontology (GO) and Kyoto Encyclopaedia of Genes and Genomes (KEGG) pathway enrichment analyses were performed. Associations between hub genes and immune infiltration and immune therapy were evaluated.

RESULTS: 3461 DEGs in TCGA-BC and 1069 DEGs in the GSE dataset were identified, with 87 overlapping differentially expressed genes between the bladder cancer and normal bladder groups. Hub genes in the tumour group were mainly enriched for cell proliferation-related GO terms and KEGG pathways, while hub genes in the normal group were related to the synthesis and secretion of neurotransmitters. PPI networks for the genes identified in the normal and tumour groups were constructed. Based on a survival analysis, CDH19, RELN, PLP1, and TRIB3 were significantly associated with prognosis ($P < 0.05$). Four hub genes were significantly enriched in the MAPK signalling pathway, VEGF signalling pathway, WNT signalling pathway, cell cycle, and P53 signalling pathway based on a gene set enrichment analysis; these genes were associated with immune infiltration levels in bladder cancer.

CONCLUSIONS: CDH19, RELN, PLP1, and TRIB3 may play important roles in the development of bladder cancer and are potential therapeutic and prognostic targets.

Introduction

Bladder cancer (BC) is a malignant tumour with the fourth highest incidence among all tumours and the first high incidence among urinary system cancers in men. According to recent cancer statistics, approximately 81400 newly diagnosed bladder cancer cases and 17980 deaths occur annually ¹. Bladder cancer can be divided into superficial and highly differentiated diseases, and patients show a good prognosis and quality of life and high mortality rates, respectively. The long-term survival outcomes are an important concern ². The superficial phenotype of bladder cancer generally corresponds to non-muscle-invasive bladder cancer (NMIBC), which accounts for 70–80% of new bladder cancer cases and is usually cured by transurethral resection of the bladder tumour, one of major challenges in the treatment of NMIBC is the high recurrence rate, which is up to 70%. Moreover, about 30% of recurrent bladder cancers will be converted into MIBC, requiring lifelong follow-up monitoring. The highly lethal type is generally classified as muscle-invasive bladder cancer (MIBC) in clinical terms, which is routinely treated by partial cystectomy and radical cystectomy. However, owing to distant metastasis in the early stage, many patients cannot undergo surgery, leading to a poor prognosis for patients with MIBC ^{3–5}. Therefore, the clinical treatment of bladder cancer usually involves a combination of surgery, chemotherapy,

radiotherapy and biotherapy. Nevertheless, progression from NMIBC towards MIBC cannot be effectively stopped. Targets for early diagnosis and treatment are urgently needed.

Owing to advances sequencing methods, genomic and chip data are becoming increasingly abundant. Many algorithms have been developed to screen for diagnostic and therapeutic targets based on expression data. In particular, weighted gene co-expression network analysis (WGCNA), a systems biology algorithm aimed at finding gene co-expression modules and exploring relationships between gene networks and clinical phenotypes^{6,7}, and differential gene expression analyses for the detection of expression differences between tumour and normal tissues and for clarifying molecular mechanisms underlying the regulation of biological processes⁸, can be used to effectively screen for potential targets for the early diagnosis and treatment of bladder cancer.

In this study, mRNA expression profiles in TCGA and GEO databases were analysed to identify differential gene co-expression, as verified in multiple databases, providing a potential basis for the clinical diagnosis and treatment of bladder cancer.

Materials And Methods

Hub gene extraction was performed strictly according to the flow chart shown in Fig. 1.

Datasets from TCGA and GEO databases

RNA-sequencing profiles and clinical characteristics for patients with bladder cancer were obtained from TCGA (<https://portal.gdc.cancer.gov/>) and the GSE13507 dataset was downloaded from GEO (<https://www.ncbi.nlm.nih.gov/gds>). The TCGA dataset included 414 bladder cancer samples and 19 normal bladder tissues and the RNAseq dataset included data for 19601 genes. Genes with low read counts were excluded as per the instructions provided with the edgeR package⁹. Therefore, only genes with a cpm (count per million) > 1 were included in subsequent analyses. A total of 14830 genes with RPKM values were selected for further analysis using the RPKM function in the edgeR package.

Additionally, the GSE13507 dataset included data for 165 primary bladder cancer samples and 9 normal tissue samples obtained using the GPL6102 platform Illumina Human-6 v2.0 Expression BeadChip. Probes were converted to gene symbols and duplicate probes for the same genes were removed via corresponding probes. Consequently, 24324 genes were included in subsequent analyses.

Differentially expressed gene identification

The R packages edge and limma were used for data collection and the identification of differentially expressed genes (DEGs) between tumour and normal tissues in the TCGA-BC and GSE13507 datasets. The DEGs were screened according to the following criteria: $|\log FC| > 1.0$ and adjusted $P < 0.05$. The pheatmap and ggplot2 packages were then used to visualise the DEGs and to generate a heatmap and volcano plot.

Detection of key co-expression modules

Weighted co-expression networks were constructed using the WGCNA package in R based on the TCGA-bladder cancer and GSE13507 gene expression profiles¹⁰. WGCNA identifies gene co-expression modules, relationships between gene networks and phenotypes, as well as core genes in networks. To establish a scale-free network and for soft thresholding power selection, pickSoftThreshold was used. Then, a_{ij} was calculated according to the following formula: $a_{ij} = |S_{ij}|^\beta$, where i and j represent genes, S_{ij} is the Pearson's correlation coefficient, β is the soft thresholding power value, and a_{ij} indicates the adjacency coefficient. The strengths of the correlations for all intergenic expression levels were calculated, and an adjacency matrix was obtained. Furthermore, a topological overlap matrix (TOM) corresponding to the dissimilarity values (1-TOM) was generated. A hierarchical clustering tree was constructed using the correlation coefficients. Different branches in the clustering tree represent different gene modules (represented by different colours in figures). Based on the weighted correlation coefficients, genes were classified according to expression patterns, and genes with similar patterns were classified into modules. Relationships between these modules and clinical parameters were evaluated to explore potential intergenic synergies related to tumorigenesis and development. The two modules with the highest positive correlation in the normal and tumour groups were selected for subsequent analyses using the TCGA-BC and GSE13507 datasets. The R package VennDiagram was used to evaluate overlapping DEGs between the two datasets and co-expressed genes in the two modules.

Functional enrichment analysis of genes

The R packages clusterProfiler and enrichplot were used for Gene Ontology (GO) and Kyoto Encyclopaedia of Genes and Genomes (KEGG) pathway enrichment analyses of genes of interest¹¹. GO uses ontologies to signify the biological relevance of genes of interest by identifying overrepresented terms in three main categories: biological processes (BP), molecular functions (MF), and cellular component (CC)¹². KEGG integrates genomic, chemical, and systemic functional information to connect genomes and biological processes¹³. Additionally, a gene set enrichment analysis (GSEA) was performed using R.

Construction of protein–protein interactions networks based on hub

genes

To obtain a more detailed and intuitive understanding of the protein interactions involving hub genes in the normal and tumour groups, the STRING (Search Tool for the Retrieval of Interacting Genes) online tool was used to visualise hub genes and construct a protein–protein interaction network¹⁴.

Validation of the prognostic value and expression of hub genes

To evaluate the clinical value of hub genes in the tumour and normal groups, TCGA data were used to detect the expression levels of these genes in bladder cancer and normal tissues. Similarly, the prognostic value of each hub gene was further evaluated using the survival package in R based on TCGA data. Only data with complete follow-up information were included in our study.

Validation of hub gene protein expression

The expression levels of proteins encoded by hub genes were verified by immunohistochemical data in the Human Protein Atlas (HPA, <https://www.proteinatlas.org/>), which provides extensive transcriptome and proteome data and includes immunofluorescence localisation information ¹⁵.

Immune infiltration database

Tumour Immune Estimation Resource (TIMER), a database for evaluating the clinical effects of different immune cells in various tumours, was used to investigate the correlation between hub genes, copy number alterations, and immune infiltration levels. Additionally, the TISIDB database was employed to investigate relationships between hub genes and immune infiltration therapy as well as subtypes in bladder cancer, and starBase was used for validation.

Results

Identification of significantly differentially expressed genes

Gene expression profiles for bladder cancer and paracancerous tissues were obtained from the GEO (<https://www.ncbi.nlm.nih.gov/geo/>) and TCGA databases(<https://portal.gdc.cancer.gov/>). After data filtering, normalisation, and ID transformation, two expression matrices were obtained from 414 bladder cancer samples and 19 adjacent bladder tissues in TCGA and 165 primary bladder cancer samples and 9 normal bladder tissues in the GSE13507 dataset. The two datasets were used to screen for DEGs based on the established criteria and results were visualised as heat maps and volcano plots, as shown in Fig. 2. In total, 3461 and 1069 DEGs met the screening criteria in the TCGA-BC and GSE13507 datasets.

Weighted gene co-expression network construction

To identify potential mechanisms underlying the development and progression of bladder cancer and to explore candidate biomarkers, the WGCNA package was used to construct a weighted gene co-expression network for both datasets (TCGA-BC and GSE13507). Seven gene modules were identified in TCGA-BC (Fig. 3) and 12 gene modules were identified in the GSE13507 dataset (Fig. 4, with different colours representing different gene modules). Subsequently, correlations between modules and clinical characters (tumour and normal) in TCGA-BC and GSE13507 were visualised by heatmaps (Fig. 3B, 4B). We identified modules that were highly associated with normal tissues (blue module in TCGA: $r = 0.85$, $p < 1e - 200$; tan module in GSE13507: $r = 0.64$, $p = 9.6e - 11$) and tumour tissues (green module in TCGA: $r = 0.7$, $p = 1.7e - 95$; yellow module in GSE13507: $r = 0.49$, $p = 3.6e - 65$). The overlap between genes in modules and DEGs in the two clinical groups (tumour and normal) were visualised by a Venn diagram.

There were 11 overlapping genes in the normal group and 76 genes in the tumour group (Fig. 5). These 87 overlapping genes were used for subsequent analyses.

Functional annotation of overlapping DEGs

The potential functions of 11 overlapping genes in the normal group and 76 genes in the tumour group were explored by GO and KEGG pathway enrichment analyses. As shown in Fig. 6, in the BP category, 11 overlapping genes in the normal group were mainly enriched in neurotransmitter uptake, long-term synaptic potentiation, and serotonin transport (Fig. 6A), and 76 genes in the tumour group were mainly related to mitotic nuclear division, nuclear division, sister chromatid segregation, and regulation of chromosome segregation (Fig. 6B), which are important processes in the development of tumours. In the CC category, overlapping genes in the normal group were related to growth cone and the site of polarised growth, and genes in the tumour group were related to chromosomal regions, condensed chromosomes, centromeric regions, and condensed chromosome kinetochores. In the MF category, genes in the normal groups were mainly involved in SNARE binding and iron ion binding, and overlapping genes in the tumour group were mainly enriched in ATPase activity and 3' to 5' DNA helicase activity. A KEGG pathway enrichment analysis showed that intersecting genes in the normal group were linked to arachidonic acid metabolism and synaptic vesicle cycle and those in the tumour group were related to mitotic nuclear division, regulation of mitotic nuclear division, and metaphase/anaphase transition of mitotic cell cycle (supplemental online Fig. 1).

PPI network construction and hub gene identification

PPI networks for the normal and tumour groups were constructed using the STRING database(<https://www.string-db.org/>) (Fig. 7) and the CytoHubba plugin was then used to calculate scores based on the MCC algorithm in order to select hub genes in the cytosol. As shown in Fig. 7A, the top 20 genes with the highest scores in the tumour group, including *KIF2C*, *NUSAP1*, *MELK*, *PBK*, *KIF20A*, *AURKA*, *NCAPG*, *TPX2*, *KIF4A*, *ASPM*, *AURKB*, *CDC20*, *CCNB1*, *BUB1B*, *CCNB2*, *CCNA2*, *BUB1*, *TOP2A*, *UBE2C*, and *TTK*, and the top 11 genes with the highest scores in the normal group. including *GPM6B*, *CYP2U1*, *SNCA*, *PLP1*, *LG14*, *RELN*, *MPZ*, *CDH19*, *GFRA3*, *COBL*, and *SNAP25*, were identified as hub genes.

Prognostic marker screening and validation of the expression of proteins encoded by hub genes

To gain further insight into the prognostic value of hub genes, the R survival package was used to analyse 31 hub genes in both normal and tumour groups. Nine genes were associated with the prognosis of bladder cancer (Fig. 8).

CDH19, *RELN*, *PLP1*, and *TRIB3* were significantly associated with prognosis ($P < 0.05$). Moreover, these four hub genes were closely related to the clinical stage of bladder cancer (supplemental online Fig. 2). The expression levels of the four hub genes were verified in TCGA-BC dataset (supplemental online

Fig. 3), *CDH19*, *RELN*, and *PLP1* levels in normal bladder tissues were significantly higher than those in bladder cancer tissues, while the expression of *TRIB3* was obviously higher in bladder cancer tissues than in normal bladder tissues. Furthermore, the protein expression level of *TRIB3* was explored using the HPA online database(<http://www.proteinatlas.org/>). As shown in supplemental online Fig. 4, the expression of *TRIB3* in normal bladder tissues was higher than that in tumour tissues, contrary to the results for RNA expression; further studies are needed to validate these findings.

Identification of hub gene-related pathways by a gene set enrichment analysis

Pathways involving four hub genes in bladder cancer were evaluated by a GSEA. The expression levels of hub genes were categorised as high and low in accordance with the cutoff value for hub genes. Then, a GSEA was performed using two datasets for four hub genes based on the normalised enrichment score (NES), nominal P-value (NOM P-val), and false discovery rate (FDR). The enrichment results for the MSigDB gene sets were significantly different according to the GSEA results (NOM P-val < 0.05, FDR < 0.05). As shown in Figure supplemental online Fig. 5A, the MAPK, VEGF, and cell adhesion molecule pathways were significantly enriched in the *CDH19*-related phenotype. The cell adhesion molecules, MAPK signalling pathway, JAK-STAT signalling pathway, and cancer-related pathways were significantly enriched in the *PLP1*-related phenotype (supplemental online Fig. 5B). The WNT signalling pathway, cancer-related in pathways, VEGF signalling pathway, and MAPK signalling pathway were significantly enriched in the *RELN*-related phenotype (supplemental online Fig. 5C). Moreover, *TRIB3* was significantly related to bladder cancer, cell cycle, and P53 signalling pathways (supplemental online Fig. 5D).

Associations between hub genes and immune infiltration or immune therapy

The relationships between hub genes and immune cell infiltration were explored using the TIMER algorithm(<http://cistrome.dfci.harvard.edu/TIMER/>)¹⁶. As shown in supplemental online Fig. 6 and supplemental online Table 1, multiple types of copy number alterations in the four hub genes, especially deletions, were significantly related to immune cells in bladder cancer, and the deletion of hub genes can reduce the level of immune infiltration in multiple immune cells. Negative correlations were detected, as shown in supplemental online Fig. 7, between hub genes and tumour purity (*CDH19*, $r = -0.26$, $p = 4.29 \times 10^{-7}$; *PLP1*, $r = -0.311$, $p = 9.63 \times 10^{-10}$; *RELN*, $r = -0.382$, $p = 3.06 \times 10^{-14}$; *TRIB3*, $r = -0.138$, $p = 8.14 \times 10^{-3}$). The statistical significance and Spearman's correlation coefficients for the relationships between *RELN*, *PLP1*, *TRIB3*, *CDH19*, and immune cell infiltration signatures are shown in supplemental online Table 1 and Table 2. The immune cell signatures included CD8 + T cells, T cells (general), B cells, monocytes, tumour-associated macrophages (TAMs), M1 macrophages, M2 macrophages, macrophages, neutrophils, natural killer cells, dendritic cells, Th1, Th2, Tfh, Th17, Treg, and T cell exhaustion (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$). Furthermore, the TISIDB database¹⁷ was used to explore the value of hub genes in bladder cancer immunotherapy. We divided bladder cancer into six groups (wound healing, IFN- γ -dominant, inflammatory, lymphocyte-depleted, immunologically quiet, and TGF- β -dominant)

according to the classification described by Thorsson et al.¹⁸. We found that *TRIB3* expression was highest in the IFN-gamma-dominant subtype, *CDH19* expression was highest in wound healing, and both *PLP1* and *RELN* levels were high in the inflammatory type (supplemental online Fig. 8). Additionally, using thresholds of $|r| > 0.5$ and $p < 0.05$, we found that both *PLP1* and *RELN* were significantly associated with *CXCL12* (sup-9A, *PLP1*, $r = 0.542$, $p < 2.2e-16$; *RELN*, $r = 0.513$, $p < 2.2e-16$), an immunoinhibitory factor. In addition, *RELN* was also strongly related to *ENTPD1* (supplemental online Fig. 9B, $r = 0.497$, $p < 2.2e-16$). The relationships between *PLP1*, *RELN*, *ENTPD1*, and *CXCL12* were verified using the starBase database (supplemental online Fig. 9D,E,F), which yielded consistent results.

Discussion

Bladder cancer is a malignant tumour with high morbidity and mortality; the overall prognosis of bladder cancer is poor. Compared with MIBC, the prognosis of NMIBC is much better; however, approximately two-thirds of cases show superficial bladder cancer relapse, despite significant progress in diagnosis and treatment. Approximately 30% of recurrent bladder cancer cases will progress to MIBC, and some patients show distant metastases, leading to an unsatisfactory 5-year survival rate¹⁹. Therefore, the early diagnosis and treatment of bladder cancer are necessary.

In this study, hub genes associated with the development of bladder cancer were identified and characterised. In total, 3461 DEGs in TCGA-BC and 1069 DEGs in the GSE13507 dataset were screened by a differential gene expression analysis. Based on DEGs identified in the two datasets, a weighted gene co-expression network was generated to identify gene modules that were highly expressed in tumour tissues and normal tissues. We identified two modules containing 4248 genes (blue module) in the TCGA dataset and 82 genes (tan module) in the GSE13507 dataset that were closely associated with normal tissues and two modules containing 641 genes (green module) in the TCGA dataset and 1061 genes (yellow module) in the GSE13507 dataset correlated with tumour tissues. We then combined the differentially expressed genes in two different modules (tumour and normal) to select candidate hub genes. A total of 11 overlapping genes in the normal group and 76 genes in the tumour group were screened. Further GO and KEGG pathway enrichment analyses indicated that hub genes in the normal group were mainly enriched in neurotransmitter uptake, long-term synaptic potentiation, and serotonin transport, and those in the tumour group were associated with cell proliferation. A PPI network was constructed and hub genes were identified (i.e., *KIF2C*, *NUSAP1*, *MELK*, *PBK*, *KIF20A*, *AURKA*, *NCAPG*, *TPX2*, *KIF4A*, *ASPM*, *AURKB*, *CDC20*, *CCNB1*, *BUB1B*, *CCNB2*, *CCNA2*, *BUB1*, *TOP2A*, *UBE2C*, *TTK*, *TRIB3*, *GPM6B*, *CYP2U1*, *SNCA*, *PLP1*, *LGI4*, *RELN*, *MPZ*, *CDH19*, *GFRA3*, *COBL*, and *SNAP25*). Among these, four genes related to prognosis, *CDH19*, *RELN*, *PLP1*, and *TRIB3*, were further identified. *CDH19*, *RELN*, and *PLP1* expression levels in normal bladder tissues were significantly higher than those in bladder cancer tissues, while the expression of *TRIB3* was higher in bladder cancer tissues than in normal bladder tissues. *TRIB3* acts as an Akt inhibitor in the liver by interacting with Akt²⁰. In bladder cancer, little is known about the regulation of *TRIB3*. However, the pattern of *TRIB3* protein expression based on data in the HPA online database was contrary to the mRNA expression pattern, and further studies are needed to

evaluate these findings. Subsequently, a GSEA was performed to identify enriched pathways. As shown in supplemental online Fig. 5, CDH19, PLP1, and RELN were both involved in the MAPK signalling pathway. MAPK, including two major isoforms, p38 α (MAPK14) and p38 β , is associated with the local inflammatory cascade in peripheral tissues²¹. PLP1 (proteolipid protein 1) is often related to nucleotide substitutions and copy number variation, leading to Pelizaeus-Merzbacher disease²². PLP1 is also involved in cancer-related pathways and the JAK-STAT signalling pathway, involved in tumorigenesis, maintenance, and metastasis in breast cancer²³. The role of PLP1 in bladder cancer has not been determined and requires further study. RELN is also associated with the WNT signalling pathway, which plays a significant role in initiation, progression, and metastasis in various types of cancers²⁴. Furthermore, as determined using the TIMER algorithm, various mutation types in the four hub genes, especially deletions, were significantly associated with immune cells in bladder cancer, and the deletion of hub genes can reduce the level of immune infiltration in multiple immune cell types (supplemental online Table 1 and Table 2). Additionally, we found that levels of both PLP1 and RELN were significantly associated with levels of CXCL12, which plays an important role in numerous physiological and pathological processes, including tumour metastasis²⁵.

However, this study had some limitations. Although our comprehensive bioinformatics analysis enabled the identification of candidate prognostic and therapeutic targets for bladder cancer, the molecular mechanisms and effectiveness of these genes in bladder cancer should be evaluated further.

Declarations

Conflict of interest

The authors including wang, chen, liao, liu, tang and mei declare no competing interests.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request. All the data analyzed in this study are included in NCBI GEO database GSE13507 (<https://www.ncbi.nlm.nih.gov/gds>) and TCGA (<https://portal.gdc.cancer.gov/>). Human Protein Atlas (HPA, <https://www.proteinatlas.org/>). STRING database(<https://www.string-db.org/>). TIMER algorithm(<http://cistrome.dfci.harvard.edu/TIMER/>).

Ethics statement

Ethical review and approval were not required for the study on human participants in accordance with the local legislation and institutional requirements. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

Author Contribution statement

AFT and HBM: conception and design. HW, JQC and XHL: analysis and interpretation of data. YL: writing of the manuscript; AFT and HBM: study supervision.

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Figures

Figure 1

Workflow chart for the study.

Figure 2

Identification of differentially expressed genes (DEGs) in the TCGA and GSE13507 bladder cancer datasets with the cut-off criteria: $|\log\text{FC}| > 1.0$ and $P < 0.05$. (A) Heatmap of DEGs in the TCGA dataset. (B) Volcano plot of DEGs in the TCGA dataset. (C) Heatmap of DEGs in the GSE13507 dataset. (D) Volcano plot of DEGs in the GSE13507 dataset

Figure 3

The relationship between modules and clinical features of bladder cancer in the TCGA-BC dataset. (A) Gene clustering tree (dendrogram) based on hierarchical clustering of adjacency-based dissimilarity values. (B) Correlations between modules and clinical features (tumour and normal).

Figure 4

Relationships between modules and the clinical features of bladder cancer in the GSE13507 dataset. (A) Gene clustering tree (dendrogram) based on hierarchical clustering of adjacency-based dissimilarity values. (B) Correlations between modules and clinical features (tumour and normal).

Figure 5

(A) Venn diagram of DEGs and genes in co-expression modules with high expression in the normal group.
 (B) Venn diagram of DEGs and genes in co-expression modules with high expression genes in the tumour group

Figure 6

(A) Gene Ontology (GO) enrichment analysis of hub genes in the normal group. (B) Gene Ontology (GO) enrichment analysis of hub genes in the tumour group.

A

B

Figure 7

Significant modules based on a protein–protein interaction (PPI) network. (A) PPI network of hub genes in the tumour group, (B) PPI network of hub genes in the normal group

Figure 8

Validation of the prognostic value of hub genes (A. GINS2; B. ZBTB4; C. TRIB3; D. CDCA5; E. ANLN; F. CHAF1B; G. PLP1; H. CDH19; I. RELN)

Supplementary Files

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